VITAMIN C ENHANCES VITAMIN E STATUS AND REDUCES OXIDATIVE STRESS INDICATORS IN SEA BASS LARVAE FED HIGH DHA MICRODIETS

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Abbreviations: α-TOH: α-tocopherol; AA: Ascorbic acid; AOE: Antioxidant enzymes; CAT: Catalase; dph: Days post hatching; DHA: Docosahexaenoic acid; EPA: Eicosapentaenoic acid; GPX: Glutathione peroxidase; IGF: insulin-like growth factors; MDA: Malonaldehyde; MUFA: Monoenics fatty acids; MyHC: Myosin heavy chain; ROS: Reactive oxygen species; SAFA: Saturated fatty acids; SOD: Superoxide dismutase; TBARS: Thiobarbituric acid reactive substances; PUFA: Polyunsaturated fatty acids; LC-PUFA: Long chain PUFA

Abstract

Docosahexaenoic acid (DHA) is an essential fatty acid necessary for many biochemical, cellular and physiological functions in fish. However, high dietary levels of DHA increase free radical injury in sea bass larvae muscle, even when vitamin E (α-tocopherol, α-TOH) is increased. Therefore, the inclusion of other nutrients with complementary antioxidant functions, such as vitamin C (ascorbic acid, AA), could further contribute to prevent these lesions. The objective of the present study was to determine the effect of AA inclusion (3600 mg/kg) in high DHA (5% DW) and α-TOH (3000 mg/kg) microdiets (diets 5/3000 and 5/3000+AA) in comparison to a control diet (1% DHA DW and 1500 mg/kg of α-TOH; diet 1/1500) on sea bass larvae growth, survival, whole body biochemical composition and thiobarbituric acid reactive substances (TBARS) content, muscle morphology, skeletal deformities and antioxidant enzymes, insulin-like growth factors (IGFs) and myosin expression (MyHC). Larvae fed diet 1/150 showed the best performance. IGFs gene expression was elevated in 5/3000 larvae, suggesting an increased muscle mitogenesis that was confirmed by the increase in the mRNA copies of MyHC. AA effectively controlled oxidative damages in muscle, increased α-TOH larval contents and reduced TBARS content and the occurrence of skull deformities. The results of the present study showed the antioxidant synergism between vitamins E and C when high contents of DHA are included in sea bass larvae diets.

Key words: DHA, vitamin E, vitamin C, muscle morphology, antioxidant enzymes, IGF, MyHC

Introduction

The importance of polyunsaturated fatty acids (PUFA) for marine fish larvae has been extensively studied during the last 20 years (1,2,3,4) in order to perfect its culture, especially in relation to eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) (1,5, 6,7). The particular structure of DHA provides this fatty acid with many important functions in fish metabolism (4), and has been demonstrated to be superior to EPA in promoting growth and conferring vitality to larvae (1,8), being preferentially incorporated into biomembranes (6,9). Besides, it has been reported that DHA content in marine fish larvae rapidly decreases during the first ten days after hatching, therefore high contents of DHA must be supplied to larvae in order to maintain the adequate levels of DHA in growing larvae (1). Due to their limited capacity to synthesize DHA and EPA from their precursors, fish require diets rich in these fatty acids (10). Nowadays, live prey substitution by compound diets is crucial for lowering costs and increasing production quality, mainly by reducing the incidence of skeletal deformities and increasing welfare in fingerlings, using a formulation having high levels of DHA, similar to those of live prey (10). However, DHA is very susceptible to attack by reactive oxygen species (ROS) due to its high unsaturation degree (11).

ROS are produced during normal cellular function (12), being beneficial or even indispensable at low concentrations in processes such as defense against microorganisms, contributing to phagocitic bactericidal activity. Fish possess enzyme systems and low-molecular-weight molecules with antioxidant functions capable of neutralizing ROS and protecting against their adverse effects (13). However, ROS generation can exceed its removal and oxidative stress can occur (14) with ROS

attacking diverse cellular components. One of the consequences of oxidative stress is the oxidative peroxidation of PUFA, known as lipid peroxidation. The high unsaturation content of these fatty acids renders them very susceptible to lipid oxidation. Thus, the high requirements of marine fish larvae for long chain PUFA (LC-PUFA), mainly DHA and EPA, makes them more prone to suffering peroxidative attack than are adults (15). Therefore, the importance of nutrition in the pro-oxidant-anti-oxidant balance process may be highly critical for fish larvae, as their high LC-PUFA contents disposes larval tissues vulnerable to oxidative stress and an increase in the content of antioxidant nutrients is essential. Among the antioxidant nutrients, vitamin E (tocopherols and tocotrienols) is the major membrane-bound lipid-soluble antioxidant (13), whereas vitamin C (ascorbic acid, AA) is an important water-soluble antioxidant which protects low density lipoproteins from oxidation and is required for the correct formation of cartilage (16). AA is easily oxidized to the unstable dehydroascorbic acid (DHAA), which is not normally detectable in plasma but may develop transiently during oxidative stress (17). The presence of sparing mechanisms between both vitamins was first hypothesized by Tappel (18). This hypothesis proposes that the oxidized α-tocopherol (α-TOH) is reduced by ascorbate, thereby regenerating α-TOH. In some fish species the presence of a vitamin C/E sparing mechanism has been suggested (19,20,21,22,23) reporting an influence on growth, tissue composition or immune responses. For instance, supplementation with 100 mg/kg of ascorbyl-2-polyphosphate to an α-TOH deficient diet in juvenile channel catfish (*Ictalurus punctatus*) decreased vertebral deformities and improved weight gain, feed intake and feed efficiency rate (23). High supplementation of ascorbate might also spare α-TOH in diets for hybrid tilapia (*Oreochromis niloticus* x *O. aureus)* as shown by the increased weight gain, feed efficiency and α-TOH concentrations. However, little is known about the effect of both

97 vitamins in preventing oxidative stress in fish larvae, when high levels of LC-PUFA are
98 administered.

99 The potentially deleterious effects of ROS are counteracted by a suite of
100 antioxidant enzymes (AOE), including radical-scavenging enzymes such as catalase
101 (CAT; EC 1.15.1.1) and superoxide dismutase (SOD; EC 1.11.1.19) or peroxidases such
102 as glutathione peroxidase (GPX; EC 1.11.1.6). In Manchurian trout larvae
103 (*Brachymystax lenok*) high lipid content microdiets stimulated the activity of AOE,
104 generally accompanied by an increase in malonaldehyde (MDA) contents (24). In
105 contrast, Mourente *et al.* (25) did not find a direct relationship between the activity of
106 AOE and the level of dietary n-3 LC-PUFA in *Dentex dentex* larvae, but decreased α-
107 TOH and increased MDA contents were found in larvae fed high n-3 LC-PUFA
108 enriched *Artemia*. Furthermore, it seems that the level of antioxidant enzymes rises with
109 larval development, whereas the level of antioxidant molecules falls (26). In mammals,
110 ROS can induce changes in gene expression during normal development (27) causing
111 defective embryo development and retardation of embryo growth (28). Similarly,
112 oxidative stress causes embryonic mortality and developmental arrest in sea urchins
113 (*Paracentrotus lividus* and *Spherechinus granularis*) larvae (29). Oxygen stress during
114 early larval stages of fish may also alter development. Somatic growth in fish is
115 regulated by insulin-like growth factors I and II (IGF-I and IGF-II), two single chain
116 polypeptides that also have a function as myogenic regulatory factors which increase
117 satellite cell proliferation and differentiation (30,31). In addition, IGFs stimulate
118 cartilage growth by the incorporation of sulphate into cartilage and also affect cell
119 differentiation, growth and proliferation (32,33). Differences in myogenesis regulation,
120 such as myosin isoform expression, have also been observed in the earliest stages and
121 during temperature acclimation (34,35,36). However, no information is available about

the effect of different dietary components on the regulation of the various components of the IGF signaling pathways, as well as their role on muscle growth in fish (37).

In previous studies we have shown the alteration of sea bass (*Dicentrarchus labrax)* larvae oxidative status when they were fed high levels of DHA (5%), with α-TOH having a limited effect in preventing alterations such as muscular dystrophy or hepatic ceroidosis (38,39). Therefore, the aim of the present study was to evaluate the combined effect of α-TOH and AA in preventing oxidative stress in sea bass larvae fed high levels of DHA in relation to larval performance or antioxidant status as well as the incidence of morphological alterations, including the expression of selected related genes.

Material and methods

Fish

The experiment was carried out at the *Grupo de Investigación en Acuicultura* facilities (Telde, Canary Islands, Spain). Sea bass, *Dicentrarchus labrax*, larvae were obtained from a natural spawning from the *Instituto de Acuicultura de Torre de la Sal* (CSIC, Castellón, Spain). Prior to starting the feeding experiment, larvae were fed enriched yeast-fed rotifers (DHA Protein Selco®, INVE, Belgium) until they reached 14 days post hatching (dph). Then, larvae (total length 8.58 ± 0.64 mm, dry body weight 0.36 ± 0.00 mg) were randomly distributed in experimental tanks (n=9) at a density of 1000 larvae/tank and were fed one of the experimental diets for 21 days, at a water temperature of 19.5 to 21.0ºC. All tanks (170 L light grey color cylindrical fibreglass tanks) were supplied with filtered sea water (34 ‰ salinity) at an increasing rate of 1.0 -

1.5 L/min along the feeding trial. Sea water entered the tank from bottom to top; water quality was tested daily and no deterioration was observed. Water was continuously aerated (125 mL/min), attaining 5-8 g/L dissolved O_2 and 60-80% saturation.

Diets

Three isonitrogenous and isolipidic experimental microdiets (pellet size < 250 μm) were prepared containing two levels of DHA, AA and α-TOH (Table 1). A low oxidation risk diet (1/1500) contained low DHA (1% DW) and α-TOH (1500 mg/kg); a high oxidation risk diet contained the highest DHA (5% DW) and α-TOH (3000 mg/kg) levels and a third diet contained high DHA (5% DW) and α-TOH (3000 mg/kg) together with increased AA (3600 mg/kg). The protein source used was squid meal defatted 3 consecutive times with a chloroform:squid meal ratio of 3:1 to allow a better control of the fatty acid profile of the microdiet. EPA, DHA, α-TOH and AA sources used in the experimental diets were EPA50 and DHA50 (CRODA, East Yorkshire, England, UK), DL- α- Tocopheryl Acetate (Sigma-Aldrich, Madrid, Spain) and Rovimix Stay-C 35 (Roche, Paris, France) respectively. Oleic acid (Merck, Darmstadt, Germany) was added to equalize the lipid content in all diets. Microdiets were prepared according to Liu *et al*. (40) by first mixing the squid powder and water soluble components, followed by lipid- and fat- soluble vitamins and, finally, warm water dissolved gelatin. The paste was pelleted and oven dried at 38ºC for 24 h. Pellets were ground and sieved to obtain particle size below 250 μm. Diets were analyzed for proximate and fatty acid composition of dry matter and kept in plastic bags under nitrogen atmosphere at -20ºC until the beginning of the experimental trial. Diets were manually supplied 14 times per day every 45 min from 9:00-19:00. Daily feed supplied

was 2, 2.5 and 3 g/tank during the first, second and third week of feeding respectively. Each diet was tested in triplicate.

Growth and survival

Final survival was calculated by individually counting live larvae at the beginning and end of the experiment. Growth was determined by measuring dry body weight (105ºC for 24 hours) and total length (Profile Projector V-12A Tokyo, Nikon) of 30 larvae/tank at the beginning, middle and end of the trial.

Biochemical analysis

All remaining larvae in each tank were washed with distilled water, sampled and kept at -80ºC for biochemical composition and TBARS analyses after 12 hours of starvation at the end of the trial. Moisture, protein (41) and lipid (42) contents of the larvae and diets were analyzed.

Total lipid fatty acid analysis

Fatty acid methyl esters (FAMEs) were obtained by transmethylation of total lipids as described by Christie (43). FAMEs were separated by GLC, quantified by FID (GC -14A, Shimadzu, Tokyo, Japan) under the conditions described in Izquierdo *et al*. (44) and identified by comparison with previously characterized standards and GLC-MS.

Determination of α-TOH *content*

α-TOH concentrations were determined in diets and larvae samples using high-pressure liquid chromatography (HPLC) with UV detection. Samples were weighed, homogenized in pyrogallol and saponified as described by McMurray *et al*. (45) for diets or according to Cowey *et al*. (46) for larvae. HPLC analysis was performed using a 150 x 4.60 mm, reverse-phase Luna 5µm C18 column (Phenomenox, California, USA). The mobile phase was 98% methanol pumped at 1.0 mL/min. The effluent from the column was monitored at a wavelength of 293 nm and quantification achieved by comparison with (+)-α-tocopherol (Sigma-Aldrich, Madrid, Spain) as external standard.

Determination of AA content

Ascorbil-2-monophosphate concentrations were determined in diets using a HPLC procedure with UV detection. The HPLC system comprised of a 150 x 4.6 mm, 5 µm particle size, Gemini C18 column fitted with a Gemini pre-column of the same material. The mobile phase consisting of phosphate buffer was delivered at a flow rate of 0.8 mL/min. Samples were dissolved with 0.4 M phosphate buffer (pH 3.0) and centrifuged at 1610 x *g* for 5 minutes at room temperature. The supernatants were kept at 4ºC until assayed. AA concentrations were determined at a wavelength of 293 nm and quantification achieved by comparison with tris(cyclohexylammonium) ascorbic acid-2-phosphate (Sigma-Aldrich, Madrid, Spain), used as a reference substance.

Measurement of thiobarbituric acid reactive substances (TBARS)

TBARS were measured in triplicate samples using a method adapted from that used by Burk *et al*. (47). Approximately 20-30 mg of larval tissues per sample were homogenized in 1.5 mL of 20% trichloroacetic acid (w/v) containing 0.05 mL of 1% BHT in methanol. To this 2.95 mL of freshly prepared 50mM thiobarbituric acid

221 solution were added before mixing and heating for 10 minutes at 100ºC. After cooling,
222 protein precipitates were removed by centrifugation (Sigma 4K15, Osterode am Harz,
223 Germany) at 2000 x *g*, the supernatant was read in a spectrophotometer (Evolution 300,
224 Thermo Scientific, Cheshire, UK) at 532 nm. The absorbance was recorded against a
225 blank at the same wavelength. The concentration of TBA-malondialdehyde (MDA)
226 expressed as nmol MDA per g of tissue was calculated using the extinction coefficient
227 0.156 μM^{-1} cm^{-1}.
228
229 **Histopathological sampling**
230
231 Thirty larvae from each tank were collected every seventh day from the
232 beginning of the feeding trial, and fixed in 10% buffered formalin for 1 or 2 days,
233 dehydrated through graded alcohols, then xylene and finally embedded in paraffin wax.
234 Six paraffin blocks containing 5 larvae per tank were sectioned at 3 µm and stained with
235 Hematoxilin and Eosin (H&E) for histopathologic evaluation (48).
236 Ten larvae per tank were fixed for 24 hours at 4ºC in 2.5% glutaraldehyde in 0.2
237 M phosphate buffer (pH 7.2). Samples were then rinsed in phosphate buffer and post-
238 fixed for 1 hour in 2% osmium tetraoxide in 0.2 M potassium ferrocyanide. Each larva
239 was then embedded in an Eppon/Araldite resin block. Serial transverse and longitudinal
240 larvae thick sections were cut at 1 µm, stained with toluidine blue and examined under
241 light microscopy (49). Thin sections were cut at 50 nm and stained with lead citrate
242 before observing with a ZEISS EM 910 transmission electron microscope (Germany) at
243 the Electron Microscope Service of University of Las Palmas de Gran Canaria.
244 Besides, 100 larvae from each tank at 35 dph were fixed in 10% buffered formalin
245 in order to perform deformity analyses. Prior to staining, larvae were measured under a

Profile Projector (Mitutoyo, PJ 3000, Japan), divided into three size classes (< 10.0 mm, 10.0-12.0 mm and >12.0 mm) and stained with Alizarin red (50) to determine bone mineralization. Larvae from the different experimental groups were stained simultaneously in order to prevent any technical variability. Deformities were classified in three different groups according to their localization: cranial deformities, lordosis, kyphosis, neural process alterations and others, including deformities such as vertebral compression or scoliosis. Cranial deformities included those found in the jaws, such as pugheadness or crossbite and opercular deformities. The axial skeleton deformities includes spine curvatures such as lordosis (ventral curvature), kyphosis (dorsal curvature) or scoliosis (lateral curvature); neural processes alterations like wrong direction or twisting and vertebral compression that comprises two alterations, the flattering of vertebral end plates and vertebral fusion. The surface corresponding to bone in whole colored larvae was visualized and quantified using a computerized image analysis package (Image-Pro Plus®, Media Cybernetics, Maryland, USA). By selecting ranges of pixel values in color images the pixels associated with red could be distinguished. The number of selected pixels was then quantified using a particle analysis operation and by counting the area of all bright objects (in pixels). Larval size was estimated by calculating the surface areas (in pixels) covered by whole stained larvae.

RNA extraction and quantitative RT-PCR

Molecular biology analyses were carried out at the University of Insubria (Varese, Italy). Total RNA was extracted from sea bass larvae (≈200 mg; pool per tank), using PureYield RNA Midiprep System (Promega, Italy). The quantity and purity of RNA

was assessed by spectrophotometer. Visualization on 1% agarose gel stained with ethidium bromide showed that RNA was not degraded. Three micrograms of total RNA was reverse transcribed into complementary DNA (cDNA) in a volume of 12 µl, including 1 µl of oligo dT16 primer (50 pmol) and 1 µl of 10 mM deoxynucleotide triphosphates (dNTPS). This mix was heated at 65ºC for 5 minutes, chilled on ice and then 4 µl of 5X reverse transcription buffer, 2 µl 0.1M DTT, 1 µl RNAse out and 1 µl of Moloney murine leukemia virus (M-MLVRT) were added. After incubation at 37ºC for 50 minutes, the reaction was stopped by heating at 75ºC for 15 minutes.

PCR primers sequences used for the PCR amplification of the cDNAs of target genes were CAT, SOD, GPX, IGF-I, IGF-II and MyHC. To perform PCR, an aliquot of 4 µl of cDNA was amplified using 25 µl GoTaq Green Master Mix (Promega, Italy) in 50 µl of final volume and 50 pmol of each designed primer.

A total of 31 PCR amplification cycles (eight touchdown) were performed for all primer sets, using an automated Thermal Cycler (MyCycler, BioRad, Italy). An aliquot of each sample was then subject to electrophoresis on a 1% agarose gel in 1X TAE buffer (Bio-Rad, Italy) and bands were detected by ethidium bromide. Samples were run together with a 100 bp±1.5 kb DNA ladder to control molecular weight of the DNA. The negative control (a reaction mixture without cDNA), confirmed the absence of genomic contamination. The PCR products from each primer set amplification were then cloned using pGEM®-T easy vector (Promega, Italy) and subsequently sequenced in both directions (T7 and SP6).

TaqMan® real time reverse transcription PCR was performed on a StepOne Real Time PCR System (Applied Biosystems, Italy) using Assays-by-Design℠ PCR primers (Applied Byosystems) and gene-specific fluorogenic probes. Primer sequences and TaqMan® probes of target genes were the following:

296

297 Target gene: Sea bass CAT

298 Forward primer: 5’- ATGGTGTGGGACTTCTGGAG - 3’

299 Reverse primer: 5’- GCTGAACAAGAAAGACACCTGATG - 3’

300 TaqMan® probe: 5’- CAGACACTCAGGCCTCA - 3’

301 Target gene: Sea bass SOD

302 Forward primer: 5’- TGGAGACCTGGGAGATGTAACTG - 3’

303 Reverse primer: 5’- TCTTGTCCGTGATGTCGATCTTG - 3’

304 TaqMan® probe: 5’- CAGGAGGAGATAACATTG - 3’

305 Target gene: Sea bass GPX

306 Forward primer: 5’- AGTTAATCCGGAATTCGTGAG - 3’

307 Reverse primer: 5’- AGCTTAGCTGTCAGGTCGTAAAAC - 3’

308 TaqMan® probe: 5’- AATGGCTGGAAACGTG - 3’

309 Target gene: Sea bass IGF-I

310 Forward primer: 5’- GCAGTTTGTGTGTGGAGAGAGA- 3’

311 Reverse primer: 5’- GACCGCCGTGCATTGG - 3’

312 TaqMan® probe: 5’- CTGTAGGTTTACTGAAATAAAA - 3’

313 Target gene: Sea bass IGF-II

314 Forward primer: 5’- TGCAGAGACGCTGTGTGG - 3’

315 Reverse primer: 5’- GCCTA CTGAAATAGAAGCCTCTGT - 3’

316 TaqMan® probe: 5’- CAAACTGCAGCGCATCC - 3’

Target gene: Sea bass MyHC

Forward primer: 5'- TGGAGAAGATGTGCCGTACTCT - 3'

Reverse primer: 5'- CGTGTCATTGATTTGACGGACATTT - 3'

TaqMan® probe: 5'- AACTGAGTGAACTGAAGACC - 3'

Data from TaqMan® PCR runs were collected with ABI´s Sequence Detector Program. Cycle threshold (Ct) values corresponded to the number of cycles at which the fluorescence emission monitored in real time exceeded the threshold limit. The Ct values were used to create standard curves to serve as a basis for calculating the absolute amounts of mRNA in total RNA. To reduce pipetting errors, master mixes were prepared to set up duplicate reactions (2 x 30 µl) for each sample.

Statistical analysis

Results are given as mean ± SD. Survival, growth, fatty acid, ossification degree and molecular biology data were tested for normality and homogeneity of variances with Levene´s test, not requiring any transformation. Chi-squared test was employed for incidence of muscular lesions, deformities and TBARS content. Survival, growth, fatty acid, ossification degree and molecular biology data were treated by one-way ANOVA. Means were compared by Duncan's test ($P<0.05$) using SPSS software (SPSS for Windows 14.0; SPSS Inc., Chicago, IL, USA, 2005). For analysis of one-way ANOVA the following general linear model was used:

$$Y_{ij} = m + D_i + e_{ij}$$

where Y_{ij} is the mean value of the tank, m is the mean population, D_i is the fixed effect of the diet and e_{ij} is the residual error.

Results

The diet containing about 1% DHA (diet 1/1500) showed higher amounts of monoenoic fatty acids than diets containing 5% DHA (5/3000 and 5/3000+AA) due to the higher oleic acid content in the former diet (Table 2). DHA contents in the diets varied from 4.58% of total fatty acids in 1/1500 diet, 24.55% in 5/3000 diet and 27.54% in 5/300+AA diet. Elevation of dietary DHA (5/3000 diets) increased n-3 PUFA and n-3 LC-PUFA fatty acids contents, as well as n-3/n-6 PUFA ratio. α-TOH levels were more than 2 times higher in diets containing 3000 mg/kg compared to the control diet (1/1500) (Table 3). AA contents were also higher in the diet supplemented with AA than in the others.

All experimental diets were well accepted by larvae. The highest total length was found in 1/1500 larvae. Increasing DHA from 1 to 5% in 5/3000 larvae, significantly reduced larvae total length, despite the α-TOH increase. However, AA levels in 5/3000+AA diet enhanced larval total length compared to 5/3000 larvae (P=0.005). Sea bass larvae survival or dry weight was not significantly different among the treatments (Table 4).

The level of lipid peroxidation-derive aldehydes, as indicated by TBARS content (nmol/g larval tissues) was lowest in larvae fed diet 1/1500 and was significantly higher in larvae fed the higher DHA content (5/3000 and 5/3000+AA diets). However, the inclusion of AA prevented the formation of hydroperoxides, observed by a decrease in TBARS levels. Thus, the lowest α-TOH content was found in larvae fed 5/3000 diet. However, an increase in dietary AA contents increased α-TOH levels in larvae fed

5/3000+AA diet. Regarding larval α-TOH contents, the elevation of dietary vitamin E in diet 5/3000, together with the increase in DHA, in comparison to diet 1/1500, did not significantly affect the α-TOH contents in the larvae (Table 4).

Fatty acid composition of the larvae (Table 5) generally reflected the fatty acid composition of the diet. Accordingly, a higher content of 18:1n-9 was observed in 1/1500 larvae. However, its retention rate regarding dietary levels was much lower in 1/1500 larvae (47.30%) than in 5/3000 (67.22%) or 5/3000+AA larvae (67.82%), balancing the monoenoic acids content among larvae fed the different dietary treatments. Equally, EPA retention was low in all larvae, especially in those fed 5/3000 (43.93%) and 5/3000+AA (43.96%) diets. The highest content of total n-3 LC-PUFA (P=0.006) was observed in larvae fed the diet supplemented with AA probably due to a higher dietary content. However, regarding 22:6n-3 content, the highest retention rate was observed in 1/1500 larvae. Similarly 20:4n-6 was highly retained in larvae fed 1/1500 diet, although higher contents were found in larvae fed diets 5/3000. In contrast to the differences in the n-3/n-6 ratio observed in diets, no differences were observed in larvae among the different treatments.

Histopathological examinations revealed the presence of lesions affecting larvae axial musculature, showing the typical features of necrotic degeneration of muscle. The incidence of muscular lesions increased with DHA dietary content (5/3000 diet; Table 4). However, inclusion of AA (5/3000+AA diet) proved to be effective in reducing incidences to less than half of those in 5/3000 larvae. More detailed features of muscular lesions could be observed on semithin and TEM sections, where muscle degeneration with the presence of hydropic vacuoles and organelles swelling within some of the affected fibers was found in larvae fed 5/3000 diet. Some fibres presented

disarrangement of the myofilaments just like myelin figures, denoting intracellular lipid oxidation (Figure 1).

Regarding skeletal morphology, among larvae measuring 10-12 mm, 28.33±1.30% of the larvae fed 1/1500 diet showed skeletal deformities, and similar values were found in those fed the 5/3000+AA diet (29.67±6.51% of the larvae anlayzed). Larvae fed the 5/3000 diet presented 33.11±5.11% deformities, but no statistical differences were detected between groups (P=0.10; Table 4). The ossification degree of 35 dph sea bass larvae was determined in terms of surface of mineralized bones per larval surface (Table 4). High dietary levels of DHA decreased the formation of mineralized bone in larvae, whereas increase in AA did not affect mineralization.

Different types of deformities were observed at the end of the experimental period, depending on the level of dietary DHA. All of the experimental groups exhibited a statistically similar percentage of kyphosis, however, no lordosis was observed in larvae fed the 1/1500 diet (Figure 2). Skull deformities were especially high in fish fed high DHA levels, although AA increase reduced the incidence of this deformity (P=0.013).

The general pattern of gene expression, excepting IGF-II, in all groups of sea bass larvae was characterized by a rapid increase between 14 and 26 dph (Figures 3 and 4). CAT gene expression was elevated in larvae fed diets containing a high content of DHA, the highest number of mRNA copies being found in larvae fed diet 5/3000+AA (P=0.027; Figure 3A). The SOD mRNA expression was also highest in 5/3000 groups (P=0.048; Figure 3B). Accordingly, GPX gene expression was quite strong in larvae fed diets containing a high level of DHA compared to larvae fed low levels (P=0.039; Figure 3C).

The IGF-I gene expression increased from 14 to 26 dph in all treatments, at day 35 showing a decrease in 1/1500 and 5/3000+AA larvae and a continuous increase in 5/3000 fed larvae (Figure 4A). Regarding IGF-II, gene expression was higher in larvae fed 5/3000 and 5/3000+AA diets than in those fed diet 1/150 throughout the trial (Figure 4B).

MyHC gene expression was low in larvae fed diet 1/1500 and significantly increased by the elevation of both α-TOH and DHA in diet 5/3000 (Figure 4C). However, increase in AA in diet 5/3000+AA significantly reduced MyHC expression to levels similar to those of 1/1500 diet.

Discussion

Marine fish larvae are subjected to high levels of oxidative stress when using inert diets due to the high content of LC-PUFA, particularly DHA, and pro-oxidants such as minerals as well as the high surface to volume ratio of these feed particles (51). Therefore, inclusion of high dietary levels of LC-PUFA to match the high requirements of marine fish larvae may call for increased dietary supplementation with antioxidants such as α-TOH to prevent oxidative damage. For instance, increasing DHA by up to 5% in diets for sea bass markedly reduced larval survival and growth and increased the incidence of muscular lesions (38). Despite an increase in dietary α-TOH from 1500 to 3000 mg/kg which partially reduced the occurrence of muscular alterations at DHA dietary levels up to 3% (38), lesions caused by the further elevation of DHA up to 5% could not be prevented by the increase in dietary α-TOH (3000 mg/kg) (39). Similarly, in the present study high levels of α-TOH (3000 mg/kg) together with high DHA (5%) were not able to counteract the adverse effects of lipid oxidation on the incidence of muscular lesions. Accordingly, these larvae showed very high levels of TBARS

indicating that their oxidative status is altered when they are fed high DHA levels even at such high dietary α-TOH levels, in agreement with our previous studies (39). Moreover, AOE expression was higher in those larvae, denoting a high antioxidant response. A compensatory induction of these endogenous antioxidants is found in animals exposed to dietary oxidative stress (52). Indeed, α-TOH contents in these larvae were not increased by the elevation of dietary α-TOH levels, suggesting a depletion of this vitamin to neutralize ROS and its limited capacity to prevent lipid peroxidation under these conditions. Similarly, increased n-3 LC-PUFA did cause depletion of α-TOH contents when this vitamin was supplemented to the diet in previous studies (51,53, 54).

In contrast, the increase in AA dietary supplementation from 1800 to 3600 mg/kg, markedly improved the protection against peroxidation, decreasing TBARS contents to less than one third, sparing vitamin E that was significantly increased in larval tissues and effectively reducing the incidence of muscular lesions. AA supplementation has also been found to reduce TBARS formation in hybrid striped bass (*Morone chrysops* ♀ x *M. saxatilis* ♂) fed diets deficient in α-TOH (21). The increased α-TOH content found in the present study when larvae were fed high levels of AA, is in agreement with studies in other fish species (20,22,51) and denotes the sparing effect of AA on α-TOH in sea bass larvae, the first vitamin recycling the second one. Thus, under dietary conditions of high LC-PUFA and α-TOH, AA dietary contents of 1800 mg/kg may not be sufficient to recycle α-TOH and prevent the high rate of ROS formation and, therefore, AA requirements may be higher than under low oxidation risk dietary situations.

AA is known to be a powerful antioxidant, by efficiently trapping peroxyl radicals in the aqueous phase before they can initiate lipid peroxidation, thereby protecting the

464 biomembranes (55). Thus, the decrease in the incidence of muscular lesions observed in
465 the present study, when high levels of AA were employed, could be due to the
466 protective effect of this antioxidant nutrient, quenching free radicals before they can
467 attack muscular membranes, in addition to recycling α-TOH. Moreover, the species
468 formed after the loss of one electron are relatively stable and fairly unreactive compared
469 to tocopheroxyl radical (56), thus preventing a pro-oxidant action. The reduced
470 incidence of muscular lesions was also accompanied by a decrease in IGF-I expression,
471 the highest IGF mRNA copies occurring in larvae fed the highest DHA and α-TOH
472 levels, which also showed the lowest growth and the highest incidence of muscular
473 lesions. These results are in line with the higher expression of IGF-I found in sea bream
474 larvae fed high DHA levels (57). On the contrary to the present study, in sea bream,
475 high IGF-I expression was correlated with high growth (57), suggesting the negative
476 effect of high DHA contents in sea bass growth in relation to the altered oxidative
477 status. A feasible explanation for the overexpression of IGF-I in larval groups with
478 higher TBARS values could be a compensatory mechanism in fish larvae to try to
479 counteract the adverse effects of ROS since IGF-I interferes with activation of apoptosis
480 in several cells and organ systems in mammals (58). For instance, an increase in IGF-I
481 has been found to suppress oxidative stress in atherosclerotic Apo-E deficient mice (59).
482 Furthermore, the IGF system can promote muscle growth and differentiation in fish, by
483 activating cell proliferation and DNA synthesis. Thus, the increase in mRNA copies of
484 IGF-I observed in larvae fed 5% DHA and 3000 mg/kg α-TOH could be also due to the
485 compensatory regeneration process carried out by satellite cells, and which was not
486 directly reflected on a growth improvement. This is supported by the results of MyHC
487 expression, as a high expression of myosin has been associated with regeneration
488 processes in sea bream after mechanical injury (60). In addition, in the present study,

489 IGF-I and MyHC expression follows a similar pattern, indicating that their biological
490 functions may be interrelated. In this sense, it is known that IGF-I overexpression
491 results in greater skeletal muscle mass in fine flounder (*Paralichthys adspersus*, 61) and
492 in mice, in which IGF-I can activate MyHC as well as other transcriptional factors (62).
493 Thus the parallel increase in IGF-I and MyHC expression observed in the present study
494 confirms that when sea bass larvae are subjected to oxidative stress, a compensatory
495 overexpression of genes related to cell/muscle proliferation occurs.

496 In the present study, AA elevation did not reduce the expression of AOE genes,
497 suggesting an antioxidant effect independently of these enzymes, but acting in parallel
498 with them to quench ROS. AA acts as a cofactor for at least eight enzymes involved in
499 the biosynthesis of collagen and carnitine, conversion of the neurotransmitter dopamine
500 to noradrenaline, metabolism of tyrosine and amidation of peptide hormones. In this
501 sense, AA acts with peptidyl-glycine alpha-amidating monooxygenase (PAM), an
502 enzyme that adds amide groups to peptide hormones, greatly increasing their stability
503 (63, 64). Thus, the antioxidant role of AA in fish might not only be reduced to trapping
504 peroxyl radicals from the aqueous phase or recycling α-TOH, but also to support the fo
505 rmation of molecules with sound antioxidant potential. More studies are required to
506 clarify the interrelations between the different components of antioxidant defenses in
507 marine fish, as well as to corroborate if the AOE activity really reflects their gene
508 expression.

509 In terms of skeletal deformities, it can be observed that a DHA increase up to 5%
510 raised the incidence of alterations in chondroid bone, such as that of the cranium,
511 whereas no differences were found in other deformities attaining intramembranous
512 bone, such as kyphosis. These results match with previous studies on sea bream larvae
513 fed high DHA rotifers (5.2% DW; 57), as ROS are known to actively destroy cartilage

514 tissue (65), therefore affecting chondroid bones with characteristics of cartilage rather
515 than directly affecting intramembranous bones. However, in the same study, in contrast
516 to the present one, the incidence of cranial deformities was reduced when high contents
517 of α-TOH were included in the rotifer enrichment media, in relation to the reduced
518 TBARS and AOE expression. In another study from our research group (66) inclusion
519 of organic selenium to diets containing high DHA and α-TOH, was enough to decrease
520 the TBARS values, but not to reduce cranial deformities, suggesting that the appearance
521 of these kind of deformity could be not only related directly to the DHA oxidation, but
522 also to the deficit of AA due to the pro-oxidant environment originated by the high
523 levels of α-TOH and LC-PUFA. Similarly, in the present study, an extra dosage of AA
524 proved to be efficient in reducing the incidence of cranial deformities when high levels
525 of DHA are included in the diets. Apart from being a potent antioxidant, AA acts with
526 three enzymes that participate in collagen hydroxylation by adding hydroxyl groups to
527 the aminoacids proline or lysine in the collagen molecules, greatly increasing stability
528 of the collagen (56). Thus, the protective effect of AA on chondroid bones could be due
529 both to its antioxidant activity and to a higher stability in the cartilage formation. Recent
530 works in sea bass larvae also showed a reduction in the incidence of cranial deformities
531 when enhanced levels of AA were included in the diet (50 mg/kg; 67). However, in the
532 same study, elevated levels of this nutrient (400 mg/kg) caused a similar percentage of
533 deformities to diets with a deficiency in AA. In the present work, the levels of AA
534 employed are much more elevated (1800 mg/kg) but it also has to be noted that levels of
535 AA higher than those required for growth are necessary to satisfy the demands of other
536 nutrients, in this case, to counteract the depletion in α-TOH caused by ROS.

537 The appearance of lordosis in fish fed high DHA and α-TOH content could be
538 related to the high IGF-I expression observed in these larvae caused by an imbalance in

the development of the musculoskeletal system, in agreement with previous studies (68). On the one hand, the high incidence of muscular lesions occurring in these larvae, may also contribute to increase the lordosis rate, as a result of the increased muscular tensions created during tissue regeneration. In this sense, Madsen and Dalsgaard (69) showed that the rainbow trout fry syndrome, characterized by muscular dystrophy among other pathologies, was associated with an increased incidence of vertebral deformities. On the other hand, IGF-II expression follows a different tendency within each dietary treatment and also as compared to IGF-I expression. These differences may support the idea that different hormonal signals and mechanisms of gene transcription control the regulation of expression of both IGF forms (70,71).

Concluding, an increased dosage of AA in microdiets for sea bass larvae containing a 5% of DHA and 3000 mg/kg of α-TOH was shown to be successful in compensating, to some extent, the effect of lipid oxidation, thereby preventing the appearance of muscular lesions, reducing cranial deformities and TBARS values, a major indicator of oxidative stress. However no counteracting effect was found on AOE expression, suggesting that other nutrients could be involved in enhancing the antioxidant defenses at such levels. Moreover, a sparing effect between AA and α-TOH seems to occur in sea bass larvae. The implication of AA in regulating other antioxidant components requires further investigation.

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Figure legends:

Figure 1: Longitudinal semithin (A) and transversal electro micrographs (B) of sea bass larvae fed 5/300 diet. (A) Damaged muscle fibres showing breakage (arrow) as well as darkening due to protein coagulation (*). (B) Affected fibre showing disarrangement of the myofilaments (arrows), swollen sarcoplasmic reticulum (SR) and myelin figures (*).

Figure 2: Skeletal deformities found in 35 dph larvae at the end of the experimental period.

Figure 3: Catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPX) expression levels measured by real-time PCR in *Dicentrarchus labrax* larvae fed diets 1/150 (♦), 5/300 (■) or 5/300+VitC (▲). mRNA copy number of each gene was normalized as a ratio to 100 ng total RNA. Values are means, with standard deviations represented by vertical bars. Mean values with unlike letters were significantly different in gene expression among the treatments at a given sampling points.

Figure 4: Insulin-like growth factors I and II (IGF-I and II) and myosin heavy chain (MyHC) expression levels measured by real-time PCR in *Dicentrarchus labrax* larvae fed diets 1/150 (♦), 5/300 (■) or 5/300+AA (▲) mRNA copy number of each gene was normalized as a ratio to 100 ng total RNA. Values are means, with standard deviations represented by vertical bars. Mean values with unlike letters were significantly different in gene expression among the treatments at a given sampling points.

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Table 1. Formulation (in % DW) of experimental diets containing two levels of DHA, vitamin E and ascorbic acid and fed to sea bass larvae for 21 days.

Dietary DHA/vitamin E	*1/1500*	*5/3000*	*5/3000+AA*
Defatted squid powder [*]	69.00	68.85	68.32
EPA[†]	2.80	1.80	1.80
DHA [†]	0.20	6.70	6.70
Oleic acid[‡]	10.00	4.50	4.50
Soy lecithin[•]	2.00	2.00	2.00
Gelatin [ǀ]	3.00	3.00	3.00
Attractants[ǂ]	3.00	3.00	3.00
Taurin[•]	1.50	1.50	1.50
Vitamin premix[§]	6.00	6.00	6.00
Mineral premix[**]	2.50	2.50	2.50
Vitamin C [††]	-	-	0.53
Vitamin E [‡‡]	-	0.15	0.15

DW, Dry weight; 1/1500, 1% DHA and 1500 g/kg vitamin E diet; 5/3000, 5% DHA and 3000 mg/kg vitamin E diet; 5/3000+AA, 5% DHA and 3000 mg/kg vitamin E diet with increased ascorbic acid content.

[*] Riber and Son, Bergen, Norway.

[†] Croda, East Yorkshire, UK.

[‡]Merck, Darmstadt, Germany.

[•]Acrofarma, Barcelona, Spain.

[ǀ]Panreac, Barcelona, Spain.

[ǂ]Attractants premix supplied per 100 g diet: Inosine-5-monophosphate, 500.0 mg; betaine, 660.0 mg; L-serine, 170.0 mg; L-phenilalanine, 250.0 mg; DL-alanine, 500.0 mg; L-sodium aspartate, 330.0 mg; L-valine, 250.0 mg; glycine, 170.0 mg.
Sigma-Aldrich

[§] Vitamin premix supplied per 100 g diet: Cyanocobalamine, 0.030; Astaxanthin, 5.00; folic acid, 5.44; pyridoxine-HCI, 17.28; thiamine, 21.77; riboflavin, 72.53; Ca-pantothenate, 101.59; p-aminobenzoic acid, 145.00; nicotinic acid, 290.16; *myo*-inositol, 1450.90; retinol acetate, 0.18; ergocalcipherol, 3.65; menadione, 17.28; α-tocopherol acetate, 150.00; ascorbyl monophosphate, 180.00.

**Mineral premix supplied g per 100 g diet: NaCl, 215.133; $MgSO_4 \cdot 7H_2O$, 677.545; $NaH_2PO_4 \cdot H_2O$, 381.453; K_2HPO_4, 758.949; $Ca(H_2PO_4) \cdot 2H_2O$, 671.610; $FeC_6H_5O_7$, 146.884; $C_3H_5O_3 \cdot 1/2Ca$, 1617.210; $Al_2(SO_4)_3 \cdot 6H_2O$, 0.693; $ZnSO_4 \cdot 7H_20$, 14.837; $CuSO_4 \cdot 5H_2O$, 1.247; $MnSO_4 \cdot H_2O$, 2.998; KI, 0.742; $CoSO_4 \cdot 7H_2O$, 10.706.

[††] Rovimix Stay-C 35, Roche, Paris, France.

[‡‡] DL-α-tocopherol acetate,Sigma-Aldrich, Madrid, Spain.

Table 2. Main fatty acids (% total of fatty acids) of the experimental diets fed to European sea bass for three weeks.

	Diet		
	1/1500	5/3000	5/3000+AA
14:0	1.54	1.26	0.78
14:1n-7	0.15	0.25	0.06
14:1n-5	0.22	0.35	0.09
15:00	0.28	0.43	0.15
15:1n-5	0.02	0.14	n.d.
16:0ISO	0.14	0.23	0.07
16:0	7.86	5.59	5.08
16:1n-7	3.59	2.26	2.01
16:1n-5	0.19	0.23	0.11
16:2n-6	n.d.	0.17	0.06
16:2n-4	0.32	0.39	0.26
17:0	1.21	0.82	0.66
16:3n-3	0.08	0.12	0.07
16:4n-3	0.09	0.13	0.10
18:0	1.29	2.29	2.18
18:1n-9+n-7	55.70	31.12	30.33
18:1n-5	0.72	0.46	0.36
18:2n-9	0.25	0.13	0.02
18:2n-6	7.40	6.99	6.71
18:2n-4	0.46	0.28	0.23
18:3n-6	0.11	0.11	0.10
18:3n-4	0.13	0.10	0.09
18:3n-3	0.72	0.83	0.82
18:3n-1	n.d.	0.04	0.04
18:4n-3	0.83	0.94	0.98
18:4n-1	0.08	0.08	0.09
20:0	0.10	0.31	0.30
20:1n-9+n-7	1.10	1.53	1.50
20:1n-5	0.05	0.12	0.11
20:2n-9	0.05	0.04	0.04
20:2n-6	0.09	0.21	0.21
20:3n-6	0.09	0.13	0.14
20:4n-6	0.71	1.57	1.62
20:3n-3	0.07	0.18	0.18
20:4n-3	0.32	0.52	0.54
20:5n-3	8.66	11.04	12.08
22:1n-11	0.17	0.51	0.53
22:1n-9	0.08	0.25	0.24
22:4n-6	0.02	0.19	0.20
22:5n-6	0.19	1.75	1.86
22:5n-3	0.32	1.29	1.42
22:6n-3	4.58	24.55	27.54
SAFA	12.28	10.70	9.16
MUFA	61.99	37.23	35.34
n-3 PUFA	15.68	39.61	43.73
n-6 PUFA	8.61	11.14	10.90
n-9 PUFA	57.19	33.10	32.17
n-3 LC-PUFA	13.96	37.58	41.76
n-3/n-6 PUFA	1.82	3.56	4.01
EPA/DHA	1.89	0.45	0.44
ARA/DHA	0.15	0.06	0.06
ARA/EPA	0.08	0.14	0.13

1/1500, 1% DHA and 1500 g/kg vitamin E diet; 5/3000, 5% DHA and 3000 mg/kg vitamin E diet; 5/3000+AA, 5% DHA and 3000 mg/kg vitamin E diet with increased ascorbic acid content.

Table 3.- Proximate composition, α-tocopherol and ascorbic acid content (mean± SD) in experimental diets fed to sea bass larvae for three weeks.

	Diets		
	1/1500	5/3000	5/3000+AA
Protein (% DW)	74.46±0.58	76.13±0.09	72.36±0.55
Ash (% DW)	5.01±0.12	5.38±0.14	5.39±0.14
Moisture (%)	10.31±0.46	9.99±0.28	9.48±0.11
Lipids (% DW)	14.98±0.31	15.80±0.02	15.94±1.05
α-tocopherol (mg/kg DW)	1410.12±38.77	3033.01±43.33	3179.72±75.69
Ascorbic acid (mg/kg DW)	1495.88±5.54	1477.44±3.29	2998.56±7.46

DW, Dry weight; 1/1500, 1% DHA and 1500 g/kg vitamin E diet; 5/3000, 5% DHA and 3000 mg/kg vitamin E diet; 5/3000+AA, 5% DHA and 3000 mg/kg vitamin E diet with increased ascorbic acid content.

Table 4.- Sea bass larvae performance and levels of lipid peroxidation products (TBARS) and vitamin E (α-tocopherol) content in sea bass larvae at the beginning and after eating the experimental diets with two levels of DHA (1 and 5%) and α-tocopherol (1500 or 3000 µg/g) and supplemented or not with ascorbic acid (1800 µg/g) for three weeks.

	Diets			
	Initial	1/1500	5/3000	5/3000+AA
Results of dietary trial				
Larval total length (mm)	8.58 ± 0.64	12.60 ± 0.93[a]	10.89 ± 1.24[c]	11.24 ± 1.08[b]
Larval dry weight (mg)	0.36 ± 0.00	1.33 ± 0.46	0.94 ± 0.05	1.01 ± 0.07
Survival (%)	-	60.51 ± 9.10	48.42 ± 4.00	47.43 ± 10.50
Incidence of muscular lesions (%)	-	17.50 ± 14.14[b]	52.63 ± 15.93[a]	20.70 ±15.62[b]
Incidence of skeletal malformations (%)	-	28.33±3.09	33.11±3.89	29.67±4.12
Ossification degree rate (%)	-	19.22±3.60[a]	15.24±4.18[b]	15.07±4.60[b]
TBARS				
NMol MDA/g dry mass	62.85±0.61	166.62±25.08[c]	2402.15±67.91[a]	846.87±94.74[b]
Vitamin E (α-tocopherol)				
mg/kg dry mass	111.45±43.26	630.24±12.39[b]	542.10±80.51[b]	757.12±44.55[a]

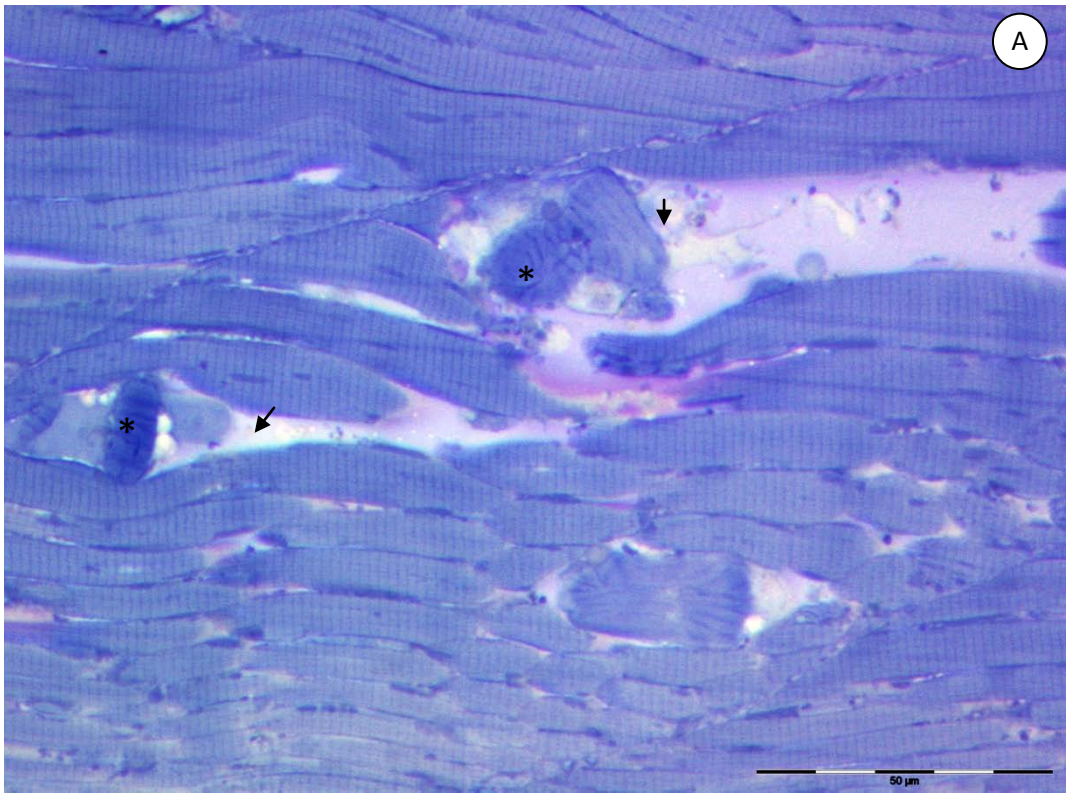
DW, Dry weight; 1/1500, 1% DHA and 1500 g/kg vitamin E diet; 5/3000, 5% DHA and 3000 mg/kg vitamin E diet; 5/3000+AA, 5% DHA and 3000 mg/kg vitamin E diet with increased ascorbic acid content.
Data are means ± SD. Values within the same row bearing different superscript letter are significantly different ($P< 0.05$)

Table 5. Main fatty acid composition of total lipids from sea bass larvae after three weeks of feeding the experimental diets (% total fatty acid).

	1/1500	5/3000	5/3000+AA
14:0	0.93±0.06	0.76±0.03	0.51±0.28
14:1n-7	0.08±0.01	0.08±0.03	0.34±0.37
14:1n-5	0.07±0.02	0.04±0.01	0.34±0.43
15:0	0.63±0.29	0.98±1.22	0.38±0.26
15:1n-5	0.12±0.10	0.09±0.06	0.79±0.23
16:0ISO	n.d.	0.11±0.01	0.28±0.25
16:0	17.51±2.44	17.61±0.25	16.32±0.26
16:1n-7	2.02±0.14	1.60±0.06	1.63±0.16
16:1n-5	0.25±0.01	0.25±0.01	0.40±0.18
16:2n-6	0.30±0.00	0.36±0.04	0.46±0.15
16:2n-4	0.93±0.40	0.96±0.15	0.89±0.12
17:0	0.91±0.10	0.80±0.03	0.81±0.08
16:3n-3	0.12±0.01	0.14±0.03	0.09±0.03
16:3n-1	0.10±0.02	0.54±0.10	0.54±0.09
16:4n-3	0.62±0.35	0.44±0.10	0.48±0.01
16:4n-1	n.d.	0.17±0.01	0.16±0.00
18:0	11.66±3.41	12.29±0.36	11.07±1.04
18:1n-9	26.35±4.87[a]	20.92±0.51[b]	20.57±0.13[b]
18:1n-7	4.85±0.29	4.44±0.39	3.99±0.23
18:1n-5	0.62±0.24	0.49±0.07	0.45±0.12
18:2n-9	n.d.	0.13±0.11	0.13±0.10
18:2n-6	4.23±0.08	3.90±0.17	3.85±0.14
18:2n-4	0.04±0.05	0.06±0.01	0.07±0.00
18:3n-6	0.43±0.01	0.38±0.04	0.33±0.01
18:3n-4	0.07±0.04	0.06±0.02	0.06±0.02
18:3n-3	0.32±0.05	0.44±0.04	0.46±0.05
18:4n-3	0.29±0.06	0.29±0.13	0.29±0.01
20:0	0.38±0.19	0.47±0.01	0.48±0.04
20:1n-9+n-7	1.83±0.0	1.77±0.06	1.80±0.02
20:1n-5	0.26±0.15	0.13±0.01	0.13±0.02
20:2n-6	0.50±0.13	0.65±0.08	0.60±0.07
20:3n-6	0.08±0.01	0.08±0.00	0.08±0.01
20:4n-6	2.38±0.04[b]	3.07±0.22[a]	3.13±0.24[a]
20:3n-3	0.12±0.09	0.15±0.04	0.13±0.00
20:4n-3	0.14±0.00	0.16±0.01	0.17±0.01
20:5n-3	5.91±1.18	4.85±0.20	5.31±0.33
22:1n-11	0.46±0.30	0.19±0.08	0.26±0.02
22:1n-9	0.26±0.15	0.26±0.06	0.27±0.05
22:4n-6	n.d.	0.11±0.02	0.13±0.04
22:5n-6	1.09±0.09	1.26±0.07	1.33±0.05
22:5n-3	0.64±0.26	0.57±0.06	0.64±0.08
22:6n-3	12.79±0.37[b]	18.04±1.19[a]	20.26±0.25[a]
Saturated	32.01±6.29	32.91±1.54	29.56±1.20
Monoenoics	36.73±4.51[a]	30.20±0.55[b]	30.56±1.44[b]
n-3 PUFA	20.94±2.23[b]	25.11±1.43[a]	27.83±0.57[a]
n-6 PUFA	8.45±0.97	9.80±0.47	9.91±0.09
n-9 PUFA	28.44±4.72[a]	23.08±0.39[b]	22.76±0.05[b]
n-3 LC-PUFA	12.92±7.45[c]	23.78±1.42[b]	26.51±0.67[a]
n-3/n-6 PUFA	2.51±0.55	2.56±0.05	2.81±0.03
EPA/DHA	0.33±0.07[b]	1.16±0.10[a]	1.11±0.17[a]
ARA/DHA	0.18±0.01[b]	0.88±0.07[a]	0.80±0.04[a]
ARA/EPA	0.54±0.04[b]	2.56±0.05[a]	3.00±0.33[a]

1/1500, 1% DHA and 1500 g/kg vitamin E diet; 5/3000, 5% DHA and 3000 mg/kg vitamin E diet; 5/3000+AA, 5% DHA and 3000 mg/kg vitamin E diet with increased

ascorbic acid content. Each value represents mean±SD. Values within the same row bearing different superscript letter are significantly different ($P<0.05$); n.d., not detected


A
50 µm

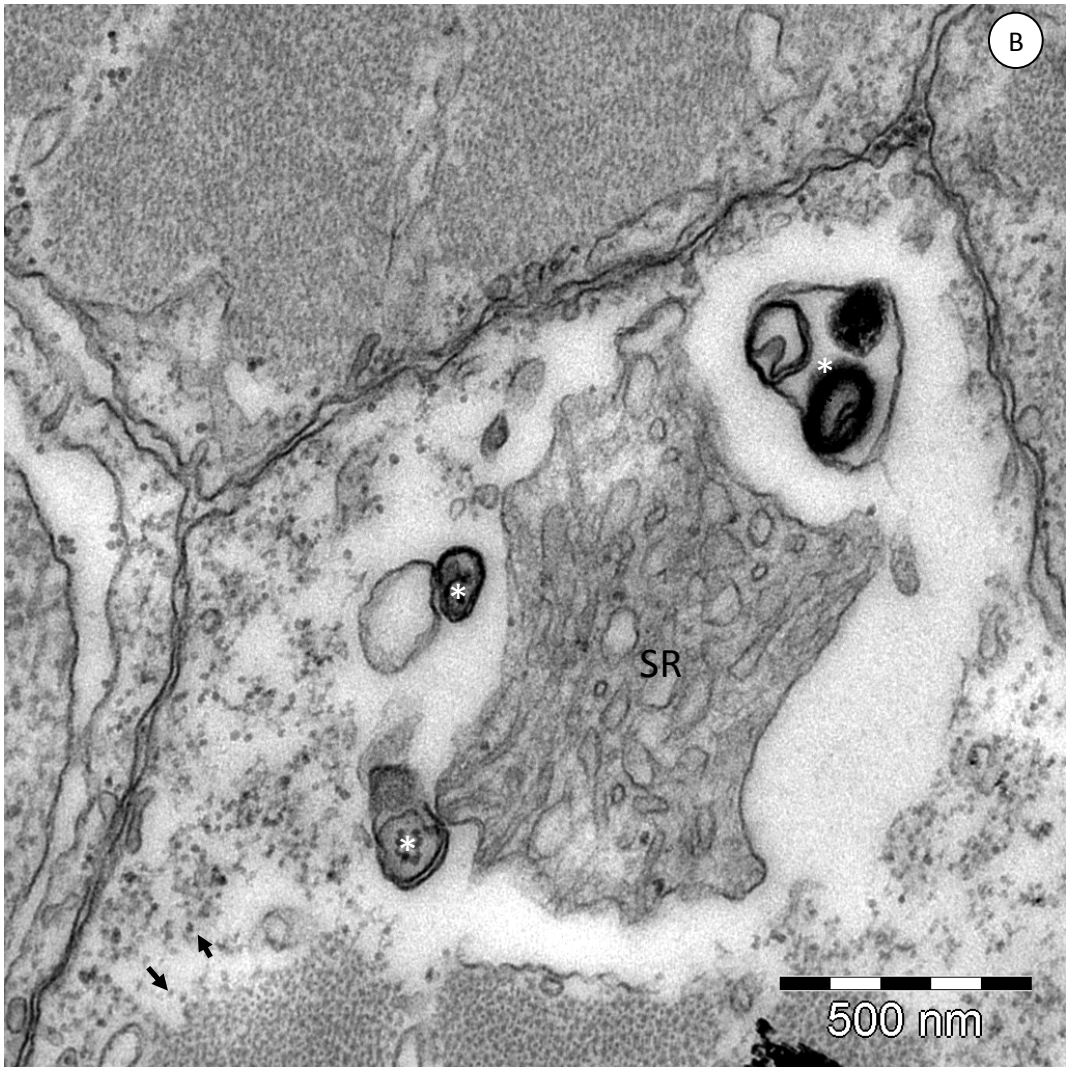

B
SR
500 nm

Figure 1.- Longitudinal semithin (A) and transversal electro micrographs (B) of sea bass larvae fed 5/3000 diet. (A) Damaged muscle fibres showing breakage (arrow) as well as darkening due to protein coagulation (*). (B) Affected fibre showing disarrangement of the myofilaments (arrows), swollen sarcoplasmic reticulum (SR) and myelin figures (*).

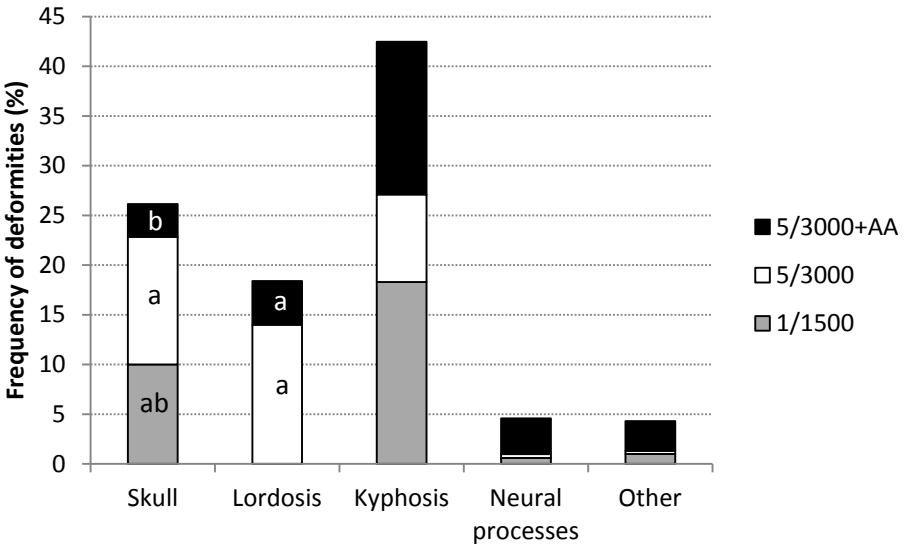


Figure 2.- Skeletal deformities found in 35 dph larvae at the end of the experimental period. Different superscript letters mean significant differences for the same type of deformity among treatments.

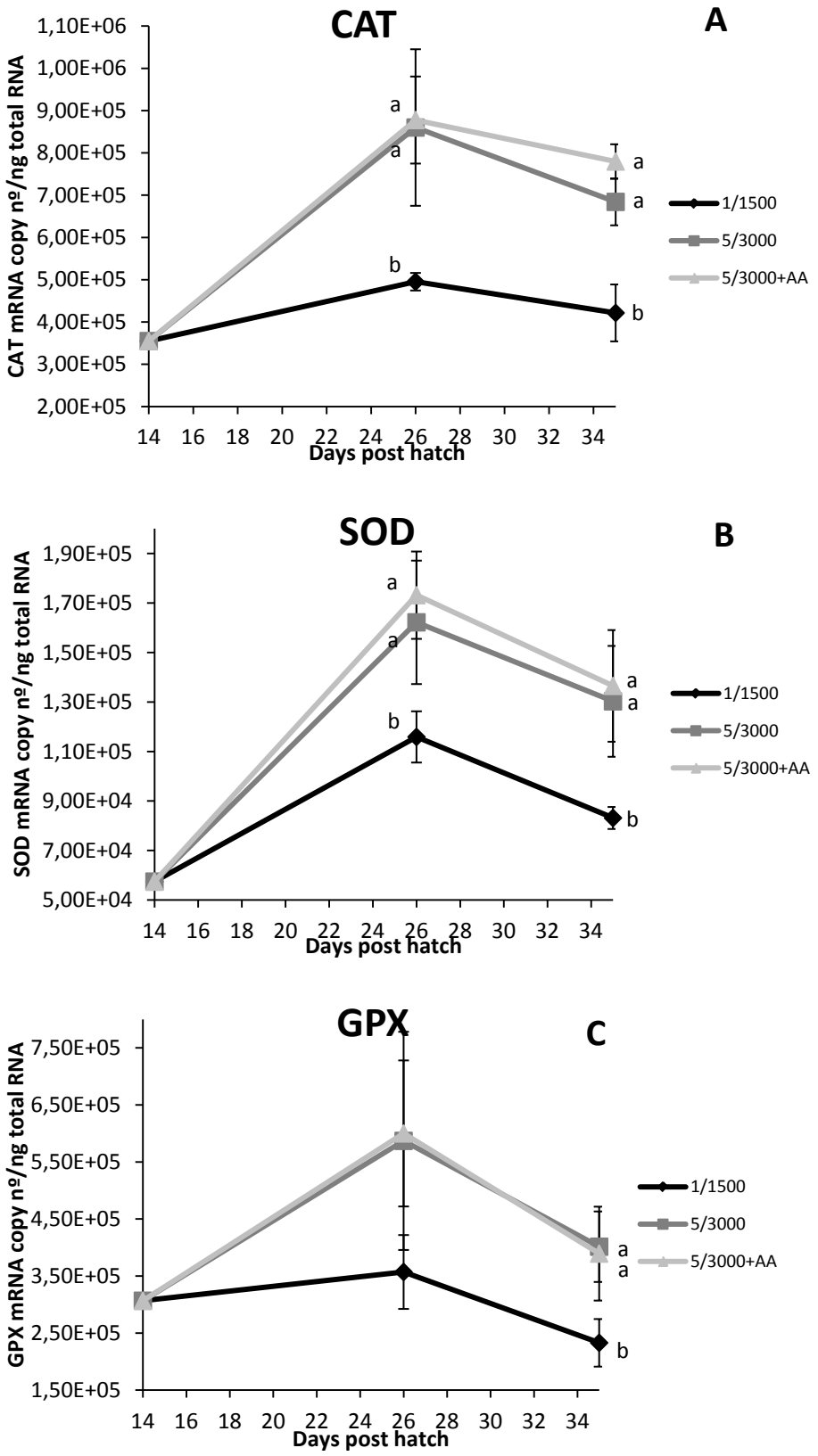
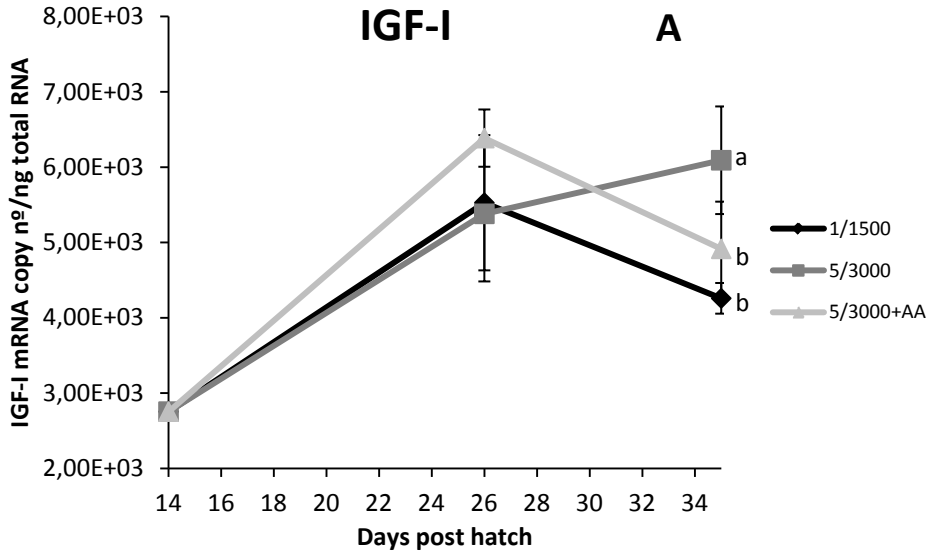
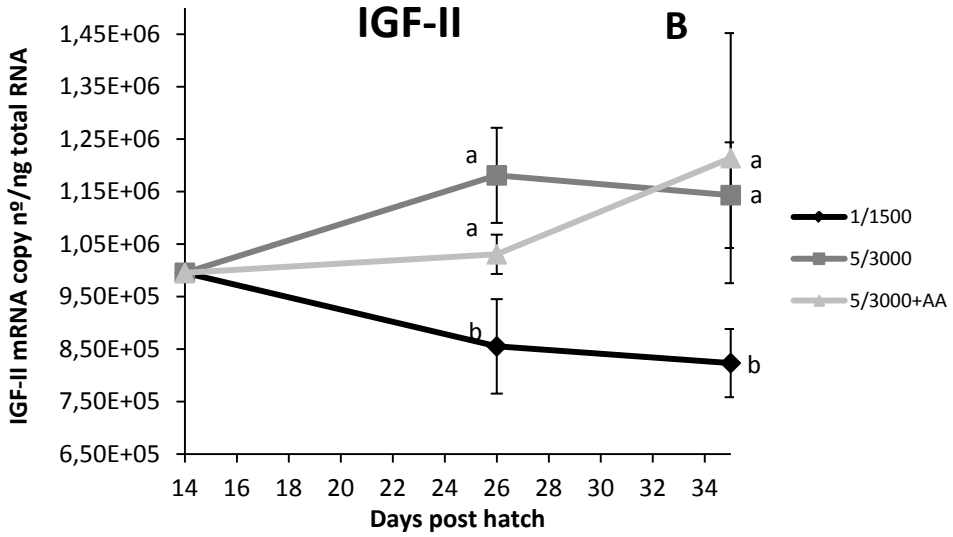


Figure 3.- Catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPX) gene expression levels measured by real-time PCR in *Dicentrarchus labrax*

larvae fed diets 1/1500 (♦;1% DHA and 1500 g/kg vitamin E), 5/3000 (■; 5% DHA and 3000 mg/kg vitamin E) or 5/3000+VitC (▲; 5% DHA and 3000 mg/kg vitamin E diet with increased ascorbic acid content). mRNA copy number of each gene was normalized as a ratio to 100 ng total RNA. Values are means, with standard deviations represented by vertical bars. Mean values with unlike letters were significantly different in gene expression among the treatments at a given sampling points.


IGF-I
A
IGF-I mRNA copy nº/ng total RNA
8,00E+03
7,00E+03
6,00E+03
5,00E+03
4,00E+03
3,00E+03
2,00E+03
14 16 18 20 22 24 26 28 30 32 34
Days post hatch
a
b
b
1/1500
5/3000
5/3000+AA


IGF-II
B
IGF-II mRNA copy nº/ng total RNA
1,45E+06
1,35E+06
1,25E+06
1,15E+06
1,05E+06
9,50E+05
8,50E+05
7,50E+05
6,50E+05
14 16 18 20 22 24 26 28 30 32 34
Days post hatch
a
a
a
a
b
b
1/1500
5/3000
5/3000+AA

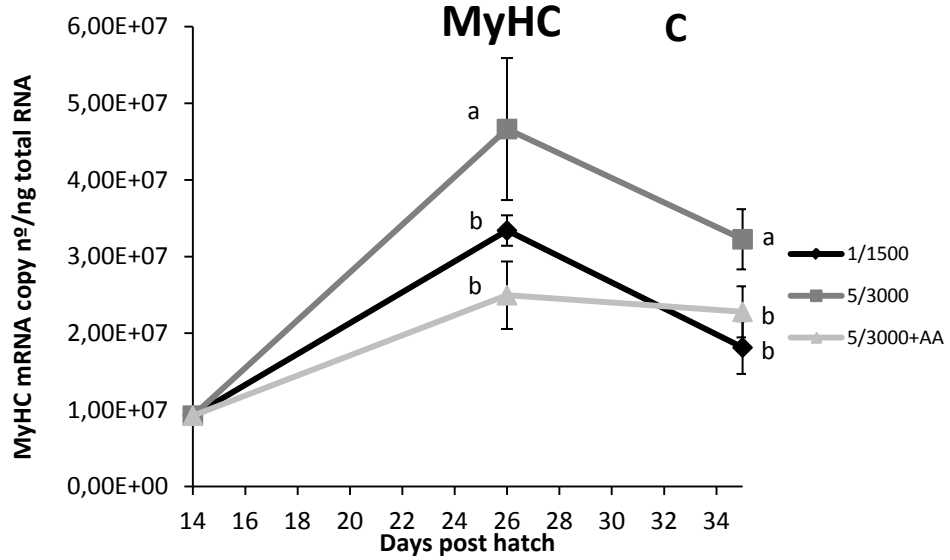

MyHC
C
MyHC mRNA copy nº/ng total RNA
6,00E+07
5,00E+07
4,00E+07
3,00E+07
2,00E+07
1,00E+07
0,00E+00
14 16 18 20 22 24 26 28 30 32 34
Days post hatch
a
b
b
a
b
b
1/1500
5/3000
5/3000+AA

Figure 4.- Insulin-like growth factors I and II (IGF-I and II) and myosin heavy chain (MyHC) gene expression levels measured by real-time PCR in *Dicentrarchus labrax* larvae fed diets 1/1500 (♦;1% DHA and 1500 g/kg vitamin E), 5/3000 (■; 5% DHA and 3000 mg/kg vitamin E) or 5/3000+VitC (▲; 5% DHA and 3000 mg/kg vitamin E diet with increased ascorbic acid content). Values are means, with standard deviations represented by vertical bars. Mean values with unlike letters were significantly different in gene expression among the treatments at a given sampling points.